Mechanism of stimulation of microsomal UDP-glucuronosyltransferase by UDP-*N*-acetylglucosamine

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We propose the existence in rat liver endoplasmic reticulum (ER) of two asymmetric carrier systems. One system couples UDP-*N*-acetylglucosamine (UDPGlcNAc) transport to UDP-glucuronic acid (UDPGlcA) transport. When UDPGlcNAc was presented at the cytosolic side of the ER, it then acted as a weak inhibitor of UDPGlcA uptake. By contrast, UDPGlcNAc produced a forceful *trans*-stimulation of microsomal UDPGlcA uptake when it was present within the lumen of the ER. Likewise, cytosolic UDPGlcA strongly *trans*-stimulated efflux of intravesicular UDPGlcNAc, whereas cytosolic UDPGlcNAc was ineffective in *trans*-stimulating efflux of UDPGlcA. A second asymmetric carrier system couples UDPGlcNAc transport to UMP transport. Microsomal UDPGlcNAc influx was markedly stimulated by UMP present inside the microsomes. Such stimulation was only apparent when microsomes had been preincubated and thereby preloaded with UMP, indicating that UMP exerted its effect on UDPGlcNAc uptake by *trans*-stimulation from the lumenal side of the ER membrane. Contrariwise, extravesicular UMP only minimally *trans*-stimulated efflux of intramicrosomal UDPGlcNAc. It is widely accepted that UDPGlcNAc acts as a physiological activator of hepatic glucuronidation, but the mechanism of this effect has remained elusive. Based on our findings, we propose a model in which the interaction of two asymmetric transport pathways, i.e. UDPGlcA influx coupled to UDPGlcNAc efflux and UDPGlcNAc influx coupled to UMP efflux, combined with intravesicular metabolism of UDPGlcA, forms a mechanism that leads to stimulation of glucuronidation by UDPGlcNAc.

INTRODUCTION

Conjugation with glucuronic acid improves the water-solubility of a large variety of endogenous and xenobiotic compounds, thereby facilitating their excretion (Dutton, 1980; Burchell and Coughtrie, 1989; Tephly and Burchell, 1990; Miners and Mackenzie, 1991). Glucuronide formation is catalysed by the UDP-glucuronosyltransferase (UGT) system, a supergene family of isoenzymes located primarily in the hepatic endoplasmic reticulum (ER) (Burchell et al., 1991).

All UGTs, which are integral membrane proteins deeply imbedded in the ER, display latency in native microsomal preparations (Dutton, 1975). It is widely believed that the catalytic centre of UGT is located within the ER lumen, and that the transferase latency may be caused by rate-limiting transport of UDP-glucuronic acid (UDPGlcA) across the microsomal membrane (Berry and Hallinan, 1974; Hallinan and de Brito, 1981; Mackenzie and Owens, 1984; Scragg et al., 1985; Vanstapel and Blanckaert, 1988b; Shepherd et al., 1989; Blanckaert et al., 1991). UDP-*N*-acetylglucosamine (UDPGlcNAc) specifically activates basal conjugation rates in latent preparations 2–5-fold. This effect, first described by Pogell and Leloir (1961), is highly dependent on structural integrity of the microsomal membrane (Winsnes, 1969; Vanstapel and Blanckaert, 1988b) and is of physiological importance (Otani et al., 1976; Blanckaert et al., 1991; Bánhegyi et al., 1993). The mechanism by which UDP-GlcNAc stimulates glucuronidation has remained unknown. A UGT model, first suggested by Winsnes (1972) and subsequently developed by Berry and Hallinan (1976), features lumenal orientation of microsomal UGT and facilitated transport of UDPGlcA across the ER membrane. These authors speculated that the UDPGlcA transporter, or 'permease', was stimulated by UDPGlcNAc during the latter's specific stimulation of glucuronidation. Such a model is consistent with the following observations. *N*-Ethylmaleimide (Winsnes, 1971) or diazobenzenesulphonate (Haeger et al., 1980) abolished the specific activation of glucuronidation by UDPGlcNAc, but did not inhibit the transferase activities themselves. Binding studies by Burchell et al. (1983) indicated that the catalytic unit of microsomal UGT was not accessible to *N*-ethylmaleimide or UDPGlcNAc. However, direct experimental evidence to support the hypothesis of Berry and Hallinan (1976) has never been presented.

Recently, we reported on a carrier system mediating translocation of intact UDPGlcA into the lumen of the ER (Bossuyt and Blanckaert, 1994a) and have characterized microsomal UDPGlcNAc transport (Bossuyt and Blanckaert, 1994b). In the present paper, we investigate whether UDPGlcNAc specifically interacts with the UDPGlcA translocation process and whether the hypothesis of Berry and Hallinan (1976) can be substantiated.

MATERIALS AND METHODS

Chemicals

UDP[^{14}C]GlcA (11.1 GBq/mmol) and UDP[^{3}H]GlcNAc (225.7 GBq/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.). UDP[^{3}H]Glc (699 GBq/mmol) was obtained from Amersham International (Amersham, Bucks, U.K.). [^{14}C]UMP (1.7 GBq/mmol), all unlabelled nucleotides, BSA (fraction V) and Hepes were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). All other chemicals were

Abbreviations used: ER, endoplasmic reticulum; RER, rough ER; UGT, UDP-glucuronosyltransferase; UDPGlcNAc, UDP-*N*-acetylglucosamine; UDPGlcA, UDP-glucuronic acid; GlcA-1-*P*, glucuronic acid 1-phosphate.

of reagent-grade quality. Aqueous solutions were prepared with deionized water.

Synthesis of [^{3}H]UDPGlcA

UDPGlcA, radiolabelled in the uridine moiety, was synthesized enzymically as described previously (Bossuyt and Blanckaert, 1994a).

Preparation and characterization of microsomes

RER (rough ER)-derived microsomes were prepared and characterized from male Wistar rat liver as described by Bossuyt and Blanckaert (1994a). Structural intactness of the microsomes, assessed by determination of mannose-6-phosphatase latency (Vanstapel and Blanckaert, 1986), was > 95% in all preparations used. All microsomes were preincubated for 150 min at 37 °C before uptake studies were started. Such preincubation was performed to remove a lumenal pool of endogenous nucleotides, which *trans*-stimulates uptake of nucleotides sugars (Vanstapel and Blanckaert, 1987; Bossuyt and Blanckaert, 1994a,b). All uptake reactions were started immediately after the 150 min preincubation. The long preincubation did not significantly alter the intactness of the microsomal vesicles, since the mannose-6-phosphatase latency of microsomes that had been preincubated for 150 min at 37 °C was still 96.5%, compared with 97.1% before preincubation.

Transport assay and analysis of microsomal breakdown of substrates

Microsomal uptake of radiolabelled nucleotide was assessed by using a rapid ultrafiltration method as previously described (Bossuyt and Blanckaert, 1994a,b). The composition of the radiolabelled components present inside the RER vesicles and the radioactive species in the extravesicular medium were determined as reported elsewhere (Bossuyt and Blanckaert, 1994a). The soluble radioactive species in either filtrate or filter extract were separated by ion-exchange chromatography as described in detail elsewhere (Bossuyt and Blanckaert, 1994a,b).

Statistics

Results are given as means ± S.D., with the numbers of observations in parentheses. Differences between means were analysed by Student's *t* test.

Protein determination

Protein was determined by the protein–Coomassie Blue dye-binding method, with BSA as calibration standard (Bradford, 1976).

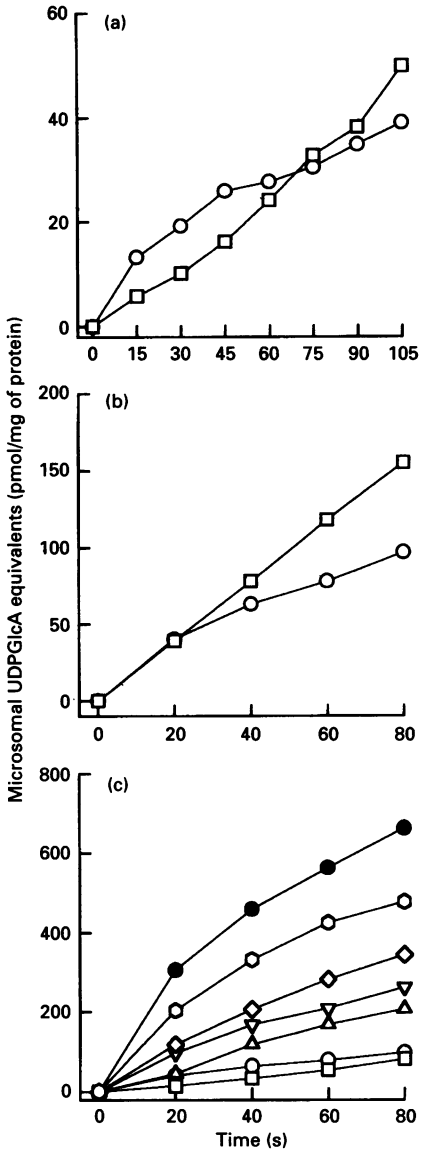

Figure 1 Effect of UDPGlcNAc on the initial rates of microsomal UDPGlcA uptake under various conditions

(**a**) Uptake of 25 μM UDP[^{14}C]GlcA in the absence (○) or presence (□) of 2 mM UDPGlcNAc added simultaneously with UDPGlcA. (**b**) Uptake of 25 μM UDP[^{14}C]GlcA in the absence (○) or presence (□) of 50 μM UDPGlcNAc added simultaneously with UDPGlcA. (**c**) Uptake of 25 μM UDPGlcA in the absence (○) or presence of 2 mM UDPGlcNAc, which had been added 0 min (□), 3 min (△), 9 min (▽), 15 min (◇), 30 min (○) or 60 min (●) before uptake was started. All data are from one representative out of at least two independent experiments using separate microsomal preparations.

RESULTS AND DISCUSSION

Effects of UDPGlcNAc on microsomal uptake of UDPGlcA

5′-Uridine nucleotide sugars, including UDPGlcNAc, have been shown to *cis*-inhibit and *trans*-stimulate translocation of UDPGlc (Vanstapel and Blanckaert, 1988a). We expected to find similar effects of UDPGlcNAc on uptake of UDPGlcA. Contrary to the expectations, we found that UDPGlcNAc, incubated with microsomes and UDP[^{14}C]GlcA, could bring about either a slight inhibition or a marked enhancement of microsomal uptake of UDPGlcA, depending on the experimental circumstances. Figures 1(a), 1(b) and 1(c) show time curves for initial uptake of radiolabel by microsomes incubated with 25 μM UDP[^{14}C]GlcA and unlabelled UDPGlcNAc under various experimental conditions. UDPGlcNAc, when added simultaneously and in a 80-fold molar excess over the 25 μM UDP[^{14}C]GlcA substrate concentration, slightly inhibited the early phase of uptake of radiolabel. Surprisingly, the initial inhibition of UDPGlcA uptake was followed, after 60 s of incubation, by a stimulation of uptake (Figure 1a). No early inhibition of ^{14}C uptake could be discerned when UDP[^{14}C]GlcA substrate and a low (50 μM) concentration of UDPGlcNAc were added simultaneously to the uptake-assay mixture. Under this condition, a stimulation of ^{14}C

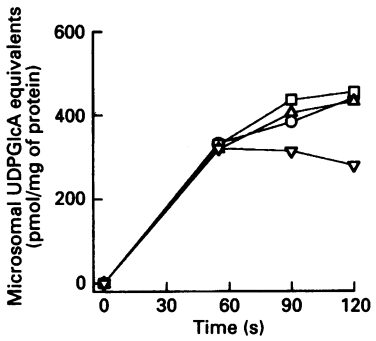

Figure 2 *trans*-Stimulation of microsomal UDP[^{14}C]GlcA efflux by unlabelled UDPGlcA, GlcA-1-*P* and GlcA

Microsomes to which 2 mM UDPGlcNAc was added during the last 60 min of the preincubation (see the Materials and methods section) were used. Such vesicles (11.4 mg/ml) were incubated with 28.5 μM UDP[^{14}C]GlcA in a total volume of 140 μl. The entry of label was monitored as a function of time. Three 30 μl portions were removed at 55 s, 90 s and 120 s. After 60 s, 20 μl of medium A (see the Materials and methods section) (○), or 20 μl from a 16 mM stock solution of UDPGlcA (▽), GlcA-1-*P* (△) or GlcA (□) was added. Values are means of three independent observations. In the condition in which unlabelled UDPGlcA was added after 60 s, vesicle-associated label determined after 120 s amounted to 66, 74 and 43% of the vesicle-associated label found under control conditions at the same time point.

uptake was seen after 20 s of incubation (Figure 1b). An enhanced uptake of radiolabel at all time points was observed when microsomes had been preincubated with a high (2 mM) concentration of UDPGlcNAc before adding the radiolabelled substrate. When the effect of UDPGlcNAc on uptake of UDPGlcA was examined as a function of the time for which the microsomes were preincubated with UDPGlcNAc before the radiolabelled substrate was added, we observed a gradual increase of the stimulation of uptake as a function of preincubation time (Figure 1c).

Based on these observations, we speculated that the unexpected stimulation of UDPGlcA uptake by UDPGlcNAc might be due to *trans*-stimulation of UDPGlcA influx by internalized UDPGlcNAc. This working hypothesis implied the presence in the ER membrane of an asymmetric carrier facilitating an exchange of extravesicular UDPGlcA for intramicrosomal UDPGlcNAc. Only high concentrations of UDPGlcNAc slightly *cis*-inhibited uptake of UDPGlcA during the time needed for entry of an amount of UDPGlcNAc sufficient to produce *trans*-stimulation of UDPGlcA uptake. Once UDPGlcNAc had accumulated in the microsomal lumen, then the *trans*-stimulation by internalized UDPGlcNAc predominated over the *cis*-inhibition at the cytosolic side. Before testing this hypothesis by *trans*-stimulation experiments, we first tried to confirm that the UDPGlcNAc-induced stimulation of radiolabel uptake in microsomes incubated with UDP[^{14}C]GlcA (i) reflected stimulation of transport of intact UDPGlcA and (ii) was caused by intact UDPGlcNAc.

Evidence that UDPGlcNAc stimulates uptake of intact UDPGlcA

UDPGlcA is rapidly and extensively broken down by highly active microsomal nucleotide pyrophosphatase and phosphatase activities. After an incubation period of 3 min we found that 29±9%, 6±1% and 65±9% ($n = 3$) of the vesicle-associated label was present in, respectively, authentic UDPGlcA, glucuronic acid 1-phosphate (GlcA-1-*P*) and GlcA when microsomes were incubated with 25 μM UDP[^{3}H]GlcA. Corresponding values for the extravesicular medium were, respectively, 95±1%, 2±1% and 3±1% ($n = 3$). Therefore it was of paramount importance to verify that, in our experiments aimed at testing the interaction between UDPGlcA and UDPGlcNAc, uptake of label reflected uptake of authentic UDPGlcA and not of a hydrolysis product, GlcA or GlcA-1-*P*. We investigated the uptake of ^{14}C label by microsomes preincubated with 2 mM UDPGlcNAc for 60 min before adding 25 μM UDP[^{14}C]GlcA. If the uptake of radioactivity represented flux of GlcA or of its 1-phosphate derivative, then the addition of an excess of unlabelled GlcA or GlcA-1-*P* would restrain the uptake of ^{14}C label. As shown in Figure 2, no inhibition was observed when an excess of these hydrolysis products was added to the assay mixture 1 min after the uptake reaction was started. By contrast, a similar concentration of unlabelled UDPGlcA did inhibit and *trans*-stimulate efflux of radiolabel present in microsomes that had been preincubated with UDP[^{14}C]GlcA. These observations demonstrate that uptake of label in the presence of the stimulatory agent UDPGlcNAc did reflect translocation of intact UDPGlcA, rather than of its hydrolysis products.

Table 1 Effect of UDPGlcNAc and its hydrolysis products on UDPGlcA uptake

Microsomes were incubated for 9 min with 2 mM UDPGlcNAc, 2 mM GlcNAc, 2 mM GlcNAc-1-*P*, 2 mM UMP or 2 mM UDP before uptake was started by addition of 25 μM UDP[^{14}C]GlcA. Initial uptake rate was estimated as described in the Materials and methods section. Values represent means±S.D. for three determinations in different microsomal preparations. When indicated by *, the mean is statistically significantly different ($P < 0.1$) from the mean of the control condition.

Condition	Uptake rate (pmol/min per mg of protein)
Control	62±24
Uridine	66±24
GlcNAc	58±28
GlcNAc-1-*P*	63±28
UMP	66±20
UDP	91±33
UDPGlcNAc	157±58*

Evidence that it is intact UDPGlcNAc that stimulates uptake of UDPGlcA

Knowing that the stimulation of UDPGlcA uptake by UDPGlcNAc was more pronounced when microsomes had been preincubated with UDPGlcNAc (Figure 1), it was imperative to demonstrate that authentic UDPGlcNAc, and not a hydrolysis product or a metabolite of this nucleotide sugar, was the stimulatory agent.

Several experimental approaches were used to tackle this question. First, we tested the effect of various hydrolytic products of UDPGlcNAc on UDPGlcA translocation (Table 1). Uptake of radiolabel by microsomes incubated with 25 μM UDP[^{14}C]GlcA was considerably stimulated by 2 mM UDPGlcNAc. By contrast, 2 mM GlcNAc, 2 mM GlcNAc-1-*P* and 2 mM-UMP exerted no effect. A small stimulation of UDPGlcA uptake was seen when vesicles had been preincubated with 2 mM UDP.

In a second approach, we examined whether UDPGlcNAc produced its effect on UDPGlcA uptake via a polyisoprenoid derivative formed from UDPGlcNAc. In the ER, UDPGlcNAc serves as a co-substrate for reactions involved in the initial steps of the dolichol (Dol) pathway, leading to the formation of Dol-

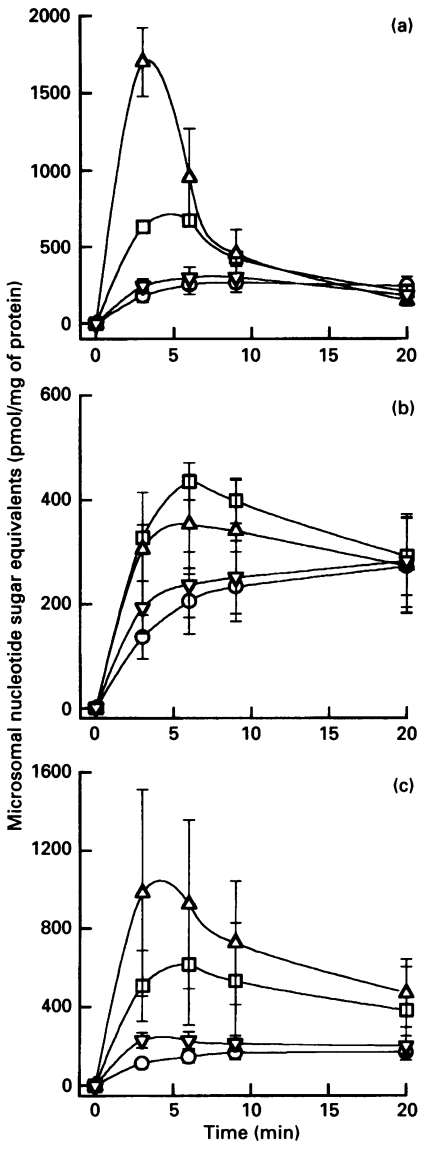

Figure 3 *trans*-Stimulation of microsomal uptake of UDPGlcA, UDPGlc and UDPGlcNAc

Microsomes were preloaded with several 5′-uridine nucleotides, after which uptake of 25 μM UDP[^{14}C]GlcA (**a**), UDP[^{3}H]Glc (**b**) or UDPGlcNAc (**c**) was studied. Preloading consisted of incubating microsomes at 37 °C for 15 min in incubation buffer without (control) (○) or with 5 mM UDPGlc (□), 5 mM UDPGlcNAc (△) or 5 mM UDPGlcA (▽). This was followed by re-isolation and washing (twice) of the vesicles by ultracentrifugation (104000 ***g*** for 30 min at 4 °C). Each data point represents the mean ± S.D. for 3–12 observations.

PP-GlcNAc and Dol-PP-$(GlcNAc)_2$ (Bossuyt and Blanckaert, 1993). We found that tunicamycin, an inhibitor of UDP-*N*-acetylglucosamine:dolichylphosphate: *N*-acetylglucosamine 1-phosphotransferase (Elbein, 1983), exerted no effect on the stimulation by UDPGlcNAc of the uptake of UDP[^{14}C]GlcA (results not shown), indicating that a GlcNAc-containing dolichol derivative did not stimulate UDPGlcA uptake.

A third potent argument supporting the idea that it is the intact UDPGlcNAc molecule that stimulated UDPGlcA uptake was obtained in studies of UDPGlcNAc metabolism. In contrast with the marked hydrolysis of UDPGlcA, metabolic breakdown of UDPGlcNAc in our preincubated microsomal preparations was limited. After an incubation period of 9 min, we found that 87 ± 2%, 8 ± 1% and 5 ± 1% ($n = 3$) of the vesicle-associated label was present in, respectively, authentic UDPGlcNAc, GlcNAc-1-*P* and GlcNAc when microsomes were incubated with 25 μM UDP [^{3}H]GlcNAc. Corresponding values for the extravesicular medium were, respectively, 94 ± 1%, 2 ± 1% and 4 ± 1% ($n = 3$).

Collectively, these results demonstrate that the enhancement of UDPGlcA uptake observed after incubation with UDPGlcNAc was due to the action of intact UDPGlcNAc and was independent of the microsomal pool of Dol-PP-GlcNAc.

trans-Stimulation of UDPGlc, UDPGlcNAc and UDPGlcA influx by 5′-uridine nucleotides

Uptake of UDPGlcA (Bossuyt and Blanckaert, 1994a), UDPGlcNAc (Bossuyt and Blanckaert, 1994b) and UDPGlc (Vanstapel and Blanckaert, 1988a) into microsomal vesicles displays *trans*-stimulation, i.e. this transport is accelerated by the presence at the *trans*-side of the membrane of a counter-substrate transported by the same carrier. We carried out a detailed examination of the relative potency of various 5′-uridine nucleotides to *trans*-stimulate UDPGlcA, UDPGlc and UDPGlcNAc uptake.

In the experiment shown in Figure 3, RER-derived vesicles were equilibrated with a high (5 mM) concentration of UDPGlc, UDPGlcA or UDPGlcNAc. After washing and resuspension of the microsomes, uptake in the vesicles was studied by using 25 μM radiolabelled substrate, i.e. UDP[^{3}H]Glc, UDP[^{14}C]GlcNAc or UDP[^{14}C]GlcA. The overshoot seen with UDP[^{14}C[GlcA as substrate was uniquely high and was most striking when vesicles had been preloaded with UDPGlcNAc (Figure 3a). Under these conditions, we found that 1704 pmol of UDP[^{14}C]GlcA equivalents/mg of protein were present in the vesicles when overshooting reached a peak. A 34-fold UDPGlcA concentration gradient across the membrane was built up under these conditions.

UDPGlcNAc was markedly less effective in *trans*-stimulating UDP[^{3}H]Glc uptake. As shown in Figure 3(b), the best agent to *trans*-stimulate UDP[^{3}H]Glc uptake was unlabelled UDPGlc. This difference between the effects on UDPGlcA uptake and UDPGlc uptake not only stresses the specificity of UDPGlcNAc as *trans*-stimulator for UDPGlcA uptake, but also underscores the exceptional magnitude of the overshoot in UDPGlcA uptake.

Intravesicular UDPGlcNAc also acted as a good *trans*-stimulator for uptake of low concentrations of radiolabelled UDP[^{3}H]GlcNAc (Figure 3c). No *trans*-stimulation of uptake of UDPGlcA, UDPGlcNAc or UDPGlc could be generated by preloading microsomes with UDPGlcA. Although intact UDPGlcA is transported into the microsomes, it is rapidly and extensively broken down to GlcA and uridine in the lumen of the microsomal vesicles. The failure to generate *trans*-stimulation by preloading with UDPGlcA is therefore most probably caused by the absence of an intramicrosomal pool of intact UDPGlcA. This is in agreement with the earlier finding that no significant endogenous glucuronidation of bilirubin could be demonstrated, even after the microsomes had been preincubated at 37 °C with 5 mM UDPGlcA. By contrast, esterification of bilirubin with glucose or xylose could be demonstrated by loading microsomes with UDPGlc or UDP-xylose (Vanstapel and Blanckaert, 1987).

Evidence for an asymmetric behaviour of the carrier mediating exchange of intravesicular UDPGlcNAc for extravesicular UDPGlcA

The preceding experiments demonstrated that the presence of intact UDPGlcNAc in the lumen of the microsomal vesicles

Table 2 *trans*-Stimulation of microsomal nucleotide sugar efflux by unlabelled nucleotide sugar

Microsomes were incubated at 37 °C at zero time with 25 μM UDP[^{14}C]GlcA, UDP[^{3}H]Glc, UDP[^{3}H]GlcNAc or [^{14}C]UMP in a total volume of 160 μl. Microsomal uptake of label was monitored as a function of time. Portions (30 μl) were removed and subjected to ultrafiltration to determine radiolabel at 55 s and 90 s. After 60 s, 20 μl of UDPGlcA, UDPGlc or UDPGlcNAc, each from a 2 mM stock solution, or 20 μl of medium A (see the Materials and methods section) (control) was added. Values represent the vesicle-associated radiolabel found after 90 s of incubation, expressed as a percentage of vesicle-associated label found after 55 s of incubation. Each data point represents the mean ± S.D. of four determinations in separate microsomal preparations, except for the *trans*-stimulation of UDPGlcNAc efflux by extravesicular UMP, which was only determined in two separate microsomal preparations. When indicated by *, the mean is statistically significantly different ($P < 0.001$) from the mean of the control condition. N.D., not determined.

Added nucleotide	Radiolabelled nucleotide...	Vesicle-associated label after 90 s (expressed as % of vesicle-associated label after 55 s)			
		UDPGlcA	UDPGlc	UDPGlcNAc	UMP
Control		114 ± 3	114 ± 2	114 ± 3	96 ± 9
UDPGlcA		87 ± 2*	102 ± 5	50 ± 4*	77 ± 18
UDPGlc		114 ± 6	82 ± 2*	87 ± 3*	76 ± 1
UDPGlcNAc		108 ± 9	98 ± 8	52 ± 6*	52 ± 6*
UMP		N.D.	N.D.	76; 72*	61 ± 5*

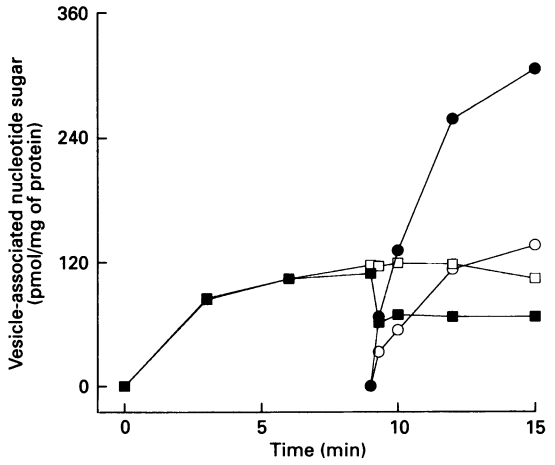

Figure 4 Influx of UDPGlcA is coupled to efflux of UDPGlcNAc

At zero time, uptake was started by addition of 25 μM UDP[^{3}H]GlcNAc (■, □) and vesicle-associated label was monitored as a function of time. After 9 min, 25 μM UDP[^{14}C]GlcA (■, ●) or medium A (see the Materials and methods section) (□) was added. An additional control condition consisted of adding 25 μM UDP[^{14}C]GlcA, but no UDP[^{3}H]GlcNAc, after 9 min (○). The data shown are from one representative out of three experiments using separate microsomal preparations.

markedly and selectively stimulated influx of UDPGlcA. We next examined whether this *trans*-stimulation depended on the side at which the two components, UDPGlcNAc and UDPGlcA, were presented. Therefore, we studied the *trans*-stimulation effect of various 5′-uridine nucleotides presented at the cytosolic side on the efflux of UDPGlc, UDPGlcA and UDPGlcNAc.

The results are presented in Table 2. Efflux of labelled substrate was markedly *trans*-stimulated by its unlabelled derivative. This was most pronounced for UDPGlcNAc, and less for UDPGlc and UDPGlcA. This difference might be partly explained by the fact that UDPGlcNAc is much more resistant to metabolic breakdown than is UDPGlcA or UDPGlc. Stimulation of UDP[^{3}H]Glc efflux by extravesicular UDPGlcA or UDPGlcNAc was limited. By contrast, UDPGlcA was an extraordinarily potent *trans*-stimulator of UDP[^{3}H]GlcNAc efflux. No *trans*-stimulation of UDP[^{14}C]GlcA (25 μM) efflux by unlabelled UDPGlcNAc or UDPGlc was found.

In conclusion, efflux of UDP[^{3}H]GlcNAc was markedly *trans*-stimulated by unlabelled UDPGlcA, whereas UDPGlcNAc was ineffective to *trans*-stimulate efflux of UDPGlcA. This indicates the presence in the ER of an asymmetric carrier system which facilitates exchange of intravesicular UDPGlcNAc for extravesicular UDPGlcA.

Influx of UDPGlcA is coupled to efflux of UDPGlcNAc

In the experiment depicted in Figure 4, microsomes were incubated with 25 μM UDP[^{3}H]GlcNAc, and vesicle-associated label was monitored as a function of time. After 9 min, 25 μM UDP[^{14}C]GlcA was added to the medium. In the time period directly after the addition of UDP[^{14}C]GlcA, a decrease in vesicle-associated ^{3}H label and an accelerated uptake of ^{14}C label was observed. This close parallelism between efflux of UDP[^{3}H]GlcNAc and influx of UDP[^{14}C]GlcA suggested direct coupling of transport of the two substrates. UDPGlcA uptake was stimulated by the presence of UDPGlcNAc at the *trans* side, i.e. in the lumen of the vesicles, even in the absence of a concentration gradient for UDPGlcNAc. For a stimulation to be possible under these conditions, one has to assume that UDPGlcNAc enters the microsomes via a transport pathway that is different from the pathway by which UDPGlcA enters the microsomes. The aim of the following experiments was to characterize the carrier which mediates entry of UDPGlcNAc into the microsomes.

Evidence that UDPGlcNAc affects the fate of UDPGlcA that is taken up by microsomal vesicles

The following series of experiments involve uptake studies in which microsomes were incubated with a mixture of [^{3}H]UDPGlcA and UDP[^{14}C]GlcA. A marked difference was seen in net uptake of these two radiolabels, depending (i) on whether UDPGlcNAc was present in the incubation mixture and (ii) on how the UDPGlcNAc was added to the incubation mixture. Specifically, UDPGlcNAc was added either simultaneously with the radiolabelled substrates or by preloading the microsomes with UDPGlcNAc. Preloading consisted of incubating the vesicles with 5 mM UDPGlcNAc so that they could take up UDPGlcNAc, with subsequent washing of the microsomes to remove the extravesicular UDPGlcNac.

Figure 5(a) shows time curves for uptake of radiolabel by control microsomes and by microsomes that had been preloaded with UDPGlcNAc. Under both conditions, the microsomes were incubated with a mixture of [^{3}H]UDPGlcA and UDP[^{14}C]GlcA. In the UDPGlcNAc-preloaded vesicles, uptake of both ^{14}C label and ^{3}H label displayed an overshoot, which was not seen under control conditions. This experiment confirms the marked *trans*-stimulation of UDPGlcA uptake by intravesicular UDPGlcNAc. In the control as well as in the UDPGlcNAc-preloaded vesicles, net uptake of ^{14}C label exceeded net uptake of ^{3}H label. This was caused most probably by efflux of ^{3}H-labelled hydrolysis products containing the uridine moiety of UDPGlcA. It should be pointed out that under these experimental conditions extensive metabolism of intravesicular UDPGlcA occurred. After incubation for 3 min of UDPGlcNAc-preloaded vesicles with 25 μM UDP[^{14}C]GlcA, intact UDPGlcA comprised only 16% of the

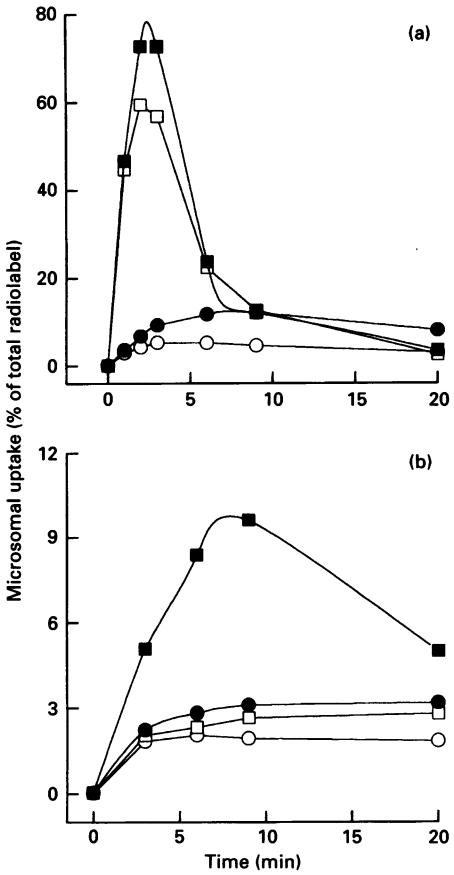

Figure 5 Uptake of a mixture of [^{3}H]UDPGlcA and UDP[^{14}C]GlcA under various conditions

(**a**) RER-derived microsomes were preincubated (see the Materials and methods section) for 150 min in the absence (○, ●) or presence (□, ■) of 5 mM UDPGlcNAc. The vesicles were then re-isolated and washed twice by ultracentrifugation (104000 *g* for 30 min at 4 °C). After resuspension, the vesicles were incubated with 25 μM UDPGlcA containing [^{3}H]UDPGlcA (□, ○) or UDP[^{14}C]GlcA (■, ●). (**b**) Microsomes were incubated with 25 μM UDPGlcA containing [^{3}H]UDPGlcA (○, □) or UDP[^{14}C]GlcA (●, ■) in the absence (○, ●) or presence (□, ■) of 50 μM UDPGlcNAc. Vesicle-associated label was monitored as a function of incubation time and expressed as percentage of total vesicle-associated radiolabel. The data shown are from one representative out of three experiments using separate microsomal preparations.

total vesicle-associated radiolabel. GlcA was the main breakdown product and contained 83% of the total vesicle-associated radiolabel. In control vesicles, which were not preloaded with UDPGlcNAc, 12% and 85% of the vesicle-associated radiolabel were respectively found in intact UDPGlcA and GlcA. After an incubation period of 3 min with 25 μM [^{3}H]UDPGlcA, 8%, 7%, 47% and 38% of the vesicle-associated radiolabel was found in, respectively, UDPGlcA, UDP, UMP and uridine. Corresponding values for the control conditions were 15%, 11%, 35% and 39%.

Figure 5(b) shows time curves for uptake of radiolabel by microsomes incubated with a mixture of [^{3}H]UDPGlcA and UDP[^{14}C]GlcA in the absence or presence of 50 μM UDPGlcNAc. In control vesicles incubated without UDPGlcNAc, net uptake of ^{3}H label was less than net uptake of ^{14}C label. When 50 μM UDPGlcNAc was present in the incubation mixture, then vesicle-associated ^{14}C label by far exceeded vesicle-associated ^{3}H label. We have demonstrated that UDPGlcNAc stimulates uptake of intact UDPGlcA (Figure 2). Therefore, we speculated that both radiolabels enter the microsomes as intact UDPGlcA, with subsequent rapid hydrolysis of the internalized UDPGlcA and efflux of radiolabelled products containing the ^{3}H-labelled uridine portion, but not of compounds comprising the ^{14}C-labelled sugar portion of UDPGlcA. That intramicrosomal hydrolysis of UDPGlcA occurred under our experimental conditions was demonstrated by h.p.l.c. analysis. After an incubation period of 3 min, authentic UDPGlcA was the main radiolabelled species in the extravesicular medium, amounting to 89±4% (n = 3) of the total radiolabel. By contrast, intact UDPGlcA comprised only 13±2% (n = 3) of the total vesicle-associated radiolabel after the same incubation period. In control microsomes incubated in the absence of UDPGlcNAc, intact UDPGlcA accounted for 95±1% (n = 3) and 29±9% (n = 3) respectively of the extravesicular and intravesicular radiolabel. By contrast with UDPGlcA, only a small fraction of the UDPGlcNAc was metabolized by the microsomes (see above).

Incubating microsomes with a mixture of [^{3}H]UDPGlcA and UDP[^{14}C]GlcA in the presence of extravesicular UDPGlcNAc resulted in intravesicular accumulation of predominantly ^{14}C label, whereas incubating UDPGlcNAc-preloaded microsomes with a mixture of [^{3}H]UDPGlcA and UDP[^{14}C]GlcA resulted in intravesicular accumulation of both radiolabels. Under both conditions, uptake of radioactivity reflected uptake of intact UDPGlcA. After uptake, extensive metabolism of the internalized UDPGlcA occurred. As no accumulation of ^{3}H could be discerned when UDPGlcNAc was supplied to the extravesicular medium, we speculated that under this situation a hydrolytic product of UDPGlcA, with the radiolabel in the uridine portion but not in the sugar moiety of the molecule, left the microsomes in exchange for extravesicular UDPGlcNAc, thereby stimulating microsomal uptake of UDPGlcNAc. Experiments were contrived to investigate the possibility that, in addition to the carrier system coupling UDPGlcA influx to UDPGlcNAc efflux, a second exchange system is present in microsomes, whereby UDPGlcNAc influx is coupled to efflux of a uridine-containing hydrolysis product.

Evidence for an asymmetric transport system mediating exchange of intravesicular UMP for extravesicular UDPGlcNAc

We investigated whether cytosolic UDPGlcNAc would, by comparison with other nucleotides, preferentially exchange with intravesicular UMP. Our results in Table 2 indicate that efflux of radiolabelled UMP was greatly stimulated by extravesicular UDPGlcNAc or unlabelled UMP. Such *trans*-stimulation effect was markedly less when UDPGlcA or UDPGlc was used as extravesicular nucleotide sugar. These findings point to specific interaction of extravesicular UDPGlcNAc with intravesicular UMP.

The asymmetry of the transport system was investigated by testing the effectiveness of various nucleotides to *trans*-stimulate efflux of intravesicular UDPGlcNAc. As shown in Table 2, efflux of radiolabelled UDPGlcNAc was affected by extravesicular UMP. However, this *trans*-stimulation caused by extravesicular UMP was less pronounced than the marked *trans*-stimulation found with unlabelled extravesicular UDPGlcA or unlabelled UDPGlcNAc. These results, on the one hand, are consistent with the existence of an asymmetric transport system mediating exchange of intravesicular UMP for extravesicular UDPGlcNAc, and on the other hand confirm the presence of a transport system exchanging intravesicular UDPGlcNAc for extravesicular UDPGlcA.

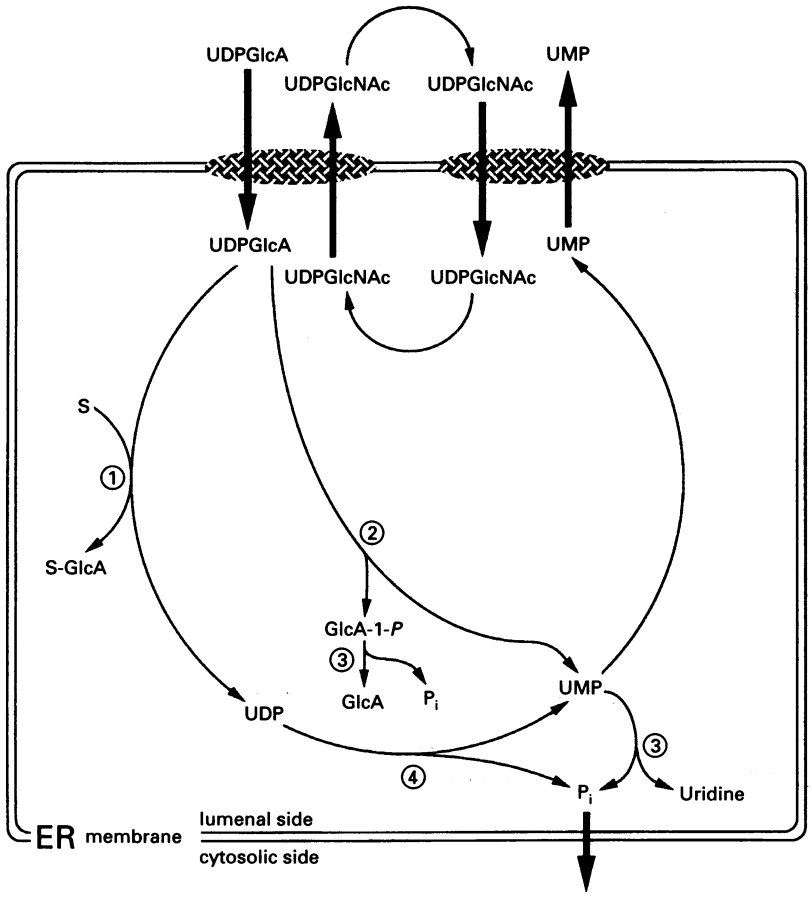

Figure 6 Model to explain stimulation of UGT by UDPGlcNAc

Enzymes: 1, UGT; 2, pyrophosphatase; 3, phosphatase; 4, nucleotide diphosphatase. Other abbreviation: S, acceptor substrate. The shaded area is the carrier protein.

In a next step, we examined whether intravesicular UMP could stimulate influx of radiolabelled UDPGlcNAc. Preincubating microsomal vesicles with 2 mM UMP for 9 min resulted in a significantly higher UDPGlcNAc uptake rate compared with the control condition, in which we tested microsomes that had not been preloaded with UMP. Uptake rates of 25 μM UDPGlcNAc were 51 ± 18 pmol/min per mg of protein ($n = 5$) and 268 ± 97 pmol/min per mg of protein ($n = 5$) respectively for the controls and for the condition in which the vesicles had been preincubated with 2 mM UMP for 9 min. Such stimulation of UDPGlcNAc uptake was only apparent when microsomal vesicles had been preloaded with UMP. When UMP was added simultaneously and in a 80-fold molar excess over the 25 μM UDPGlcNAc substrate concentration, then no effect of UMP on microsomal uptake of UDP[^{3}H]GlcNAc could be observed. Under these conditions, the initial UDPGlcNAc uptake rate was 51 ± 17 pmol/min per mg of protein ($n = 3$). These findings indicated that UMP exerted its effects from the lumenal side of the membrane.

Efflux of UMP is specifically coupled to influx of UDPGlcNAc

The flux of UMP out of the microsomes is specifically coupled to the inward flux of UDPGlcNAc, and not to the inward flux of UDPGlcA. This is evidenced by the following two observations. UDPGlcA was not effective in *trans*-stimulating UMP efflux (Table 2), and no enhanced uptake of UDPGlcA could be generated by preincubating microsomes with UMP (Table 1). These findings provide evidence that there is no direct coupling between UDPGlcA influx and UMP efflux, and underscore the role of UDPGlcNAc as a shuttle compound in helping UDPGlcA to enter the microsomes and in removing UMP from the ER lumen.

General conclusions

From our results, we postulate the model presented in Figure 6 to explain the stimulation of microsomal glucuronidation by UDPGlcNAc. The model encompasses at least two separate transport pathways, both of which effect an asymmetric exchange: UDPGlcA influx coupled to UDPGlcNAc efflux and UDPGlcNAc influx coupled to UMP efflux.

UDPGlcNAc, by *trans*-stimulation, enhances the uptake of UDPGlcA in the microsomes and delivers the co-substrate to the catalytic centre of UGT. UDP, formed by UGT, is hydrolysed to UMP and P_i by nucleotide diphosphatase. In the absence of an acceptor substrate, internalized UDPGlcA is rapidly and extensively metabolized by the action of microsomal pyrophosphatase. This results in the formation of GlcA-1-*P* and UMP. GlcA-1-*P* is further hydrolysed to GlcA and P_i. The P_i may leave the microsomal lumen via a P_i transporter (Burchell, 1990). UMP is transported out of the microsomes in exchange

for UDPGlcNAc, which enters the microsomes. The internalized UDPGlcNAc then becomes available as counter-substrate for UDPGlcA influx. In this way UDPGlcNAc shuttles between the lumenal side and the cytoplasmic side of the microsomes, once in exchange with UMP to enter the microsomes, and once in exchange with UDPGlcA to leave the microsomes. The interaction of two asymmetric transport systems together with intravesicular metabolism of UDPGlcA forms the mechanism of the UDP-GlcNAc-induced stimulation of glucuronidation.

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